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Research report

# Proteomic characterisation of neuronal sphingolipid-cholesterol microdomains: role in plasminogen activation

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**Abstract**

Sorting of certain membrane proteins requires a mechanism involving rafts, protein–lipid complexes enriched in glycosphingolipids and cholesterol. These microdomains remain at the plasma membrane of different cell types and play a role in signal transduction. Although recent reports have begun to describe molecules associated with rafts, their protein composition remains largely unknown, especially in neuronal cells. To address this question, we have purified detergent-insoluble raft fractions (DRMs) from primary cultures of hippocampal neurons. Bidimensional gel analysis and pharmacological raft lipid manipulation allowed the identification of neuronal raft proteins and their characterisation by MALDI-TOF analysis. Enolases were found among the proteins identified and functional studies demonstrate their participation in plasminogen binding. We also show the specific enrichment in rafts of several other plasminogen binding molecules and the exclusive activation of plasminogen to the protease plasmin in these microdomains. These observations suggest that neuronal rafts may play, in addition to intracellular signaling, a role in extracellular/membrane protein proteolysis.


*Theme:* Cellular and molecular biology

*Topic:* Membrane composition and cell-surface macromolecules

*Keywords:* Membrane raft; Neurons; Enolase; Plasmin

## 1. Introduction

Protein–lipid rafts were first described in polarized epithelial cells [43]. They are complexes formed during protein processing in the Golgi apparatus where certain proteins interact with specific lipids. This renders the proteins resistant to extraction with detergents at 4 °C and confers on them the ability to float in gradient centrifugation to light density fractions corresponding to the buoyant density of the lipids [5,8,46]. The importance of these complexes in membrane trafficking has been extensively documented. Inhibition of the main raft lipids, sphingomyelin and cholesterol, or deletions of protein domains responsible for the interaction with lipids, randomizes the distribution of epithelial apical proteins [17,26]. Our previous results demonstrated that hippocampal neurons in vitro, highly polarized cells developing dendritic and axonal arbors similar to their counterparts in situ [7], also use rafts to sort certain axonal membrane proteins [21]. Moreover, the lack of raft formation in developing neurons is consistent with the absence of polarized delivery at early developmental stages [22]. These studies suggested that appropriate targeting for certain axonal proteins requires a developmental increase in the levels of sphingomyelin to guarantee the interaction with these proteins and, consequently, raft formation.

It has been shown that rafts are not simply complexes needed for the correct sorting of membrane proteins but that they also remain at the cellular plasma membrane [10,47]. Signaling molecules have been identified in rafts and their function depends on raft integrity indicating a role as platforms for cell signaling for these microdomains

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[44]. Caveolins, scaffold proteins that bind cholesterol, were also found to be part of epithelial sphingolipid-cholesterol microdomains but they have not been identified in other cell types such as neuroblastoma cells [12], lymphocytes [9] or hippocampal neurons [35]. This is an indication that different cell types may have distinct proteins associated with rafts, permitting the exertion of different functions. In this work we have isolated detergent-insoluble raft fractions (DRMs) from primary hippocampal neurons in culture. This has allowed systematic characterisation of the protein composition of neuronal rafts, providing insights into the possible involvement of these complexes in specific neuronal functions.

## 2. Materials and methods

### 2.1. Cell culture

Cultures of hippocampal neurons were prepared from the brains of 18-day-old rat embryos as described [13]. These neurons survive for several weeks and undergo full polarization when cultured in serum-free medium ($N_2$). For our experiments cells were kept in culture for 4–5 days (immature stage 4 neurons) or 8–15 days (mature stage 5 neurons).

### 2.2. Detergent extraction, raft isolation and radiolabeling

Stage 4 and 5 neurons were extracted for 1 h on ice in 1% Triton X-100 in buffer A: 25 mM MES pH 7.00, 5 mM DTT, 2 mM EDTA and CLAP (25 μg/ml each of chymostatin, leupeptin, antipain and pepstatin A). The extracts (200 μg protein/ml buffer) were mixed with 60% Optiprep™ (Nycomed) to reach a final concentration of 40% and overlayed in an SW40 centrifugation tube (Beckman) with a step gradient of 30 and 5% Optiprep™ in buffer A. After a 5-h centrifugation at 35 000 rpm, the raft fraction was obtained from the interphase 30–5% Optiprep™.

In the case of radioactive labeled extracts, 500 000 neurons per point were incubated with 200 μCi/ml [$^{35}$S]methionine for 12 h. Same amount of total protein was detergent extracted and floated in optiprep gradients. Samples were then analysed in 2D gels with a standard minigel system (Bio-Rad).

### 2.3. Sphingomyelin depletion

Fumonisin B1 (FB1; Sigma) was added from a 1 mM stock solution in 20 mM HEPES, pH 7.4 to neurons, 24 h after plating. FB1 was subsequently added every 48 h each time reaching a final concentration of 25 μM. After 8 days of treatment cells were processed by radiolabeling followed by detergent extraction, centrifugation in an Optiprep™ gradient and analysis in 2D gels. This treatment was shown to alter raft protein detergent insolubility and raft mediated sorting in cultured hippocampal neurons [21].

### 2.4. Cholesterol depletion

After 5 days in culture, 4 μM lovastatin (kindly provided by Renate Luedecke, MSD Sharp & Dohme, Haar, Germany) and 0.25 mM mevalonate (Sigma) were added to the neuronal medium. Lovastatin in the presence of a low amount of mevalonate inhibits cholesterol biosynthesis [2] while allowing synthesis of non-sterol products [4]. Neurons were grown for 4 more days and were then metabolically labeled and further incubated with 5 mM methyl-β-cyclodextrin (Sigma) for 15 min at 37 °C. The samples were collected after 25 min in $N_2$ medium containing lovastatin and mevalonate. They were then processed as described for FB1. Cholesterol removal was monitored by fluorescence microscopy in neurons plated in coverslips, using the cholesterol binding antibiotic filipin as in Simons et al. [45].

### 2.5. NBD-C6-Ceramide treatment

Immature stage 4 neurons were treated with 0.5 μm NBD-C6-Ceramide (Molecular Probes) for 24 h. Ceramide was added to the cultures in ethanol (reaching a final concentration of 1% ethanol). Then, cells were washed, radiolabeled in the presence or absence of ceramide, detergent extracted and subsequently centrifuged in an Optiprep™ step gradient before analysis in a 2D gel.

### 2.6. Protein sequencing analysis

Proteins were excised from the gel and in-gel digested in trypsin as described in Schevchenko et al. [40]. Proteins were identified by peptide mass mapping. The masses of the tryptic peptides of a protein were determined and a non-redundant database (nrdb) was searched for proteins that produce, upon digestion, peptides corresponding in mass to the measured set. The peptide masses were determined on a MALDI Bruker REFLEX instrument (Bruker Franzen Analytik, Bremen, Germany) and database searches were done in nrdb using PeptideSearch with mass accuracies of 70 ppm [25].

### 2.7. Immunodetection by Western blot and slot-blot

Monoclonal anti-rat transferrin receptor (Pharmingen), monoclonal anti-flotillin 1 (BD Transduction) and monoclonal antibody to mitochondria (Biodesign International) were used to characterize the optiprep fractions by Western blot of 1D gels. The following antibodies were used to

detect plasminogen binding molecules: polyclonal anti-α enolase (Biogenesis); polyclonal anti-HMG1 (amphoterin; PharMingen International); and monoclonal anti-annexin II (Transduction Laboratories). Plasmin was detected by a polyclonal antibody against rat plasminogen (Biogenesis). Species-specific peroxidase conjugated secondary antibodies and the ECL method (Amersham) were subsequently used. We measured GM1 by slot-blot using cholera toxin subunit B peroxidase linked (Sigma).

### 2.8. Immunofluorescence of surface membrane proteins

For the localization of neuronal specific enolase (γ enolase) in the neuronal surface, the cells were incubated with the monoclonal anti γ enolase (Quartett, Biogenesis) diluted in culture medium for 8 min at 37 °C and 5% $CO_2$. The cells were fixed with 4% PFA and without permeabilization they were incubated with rhodamine conjugated anti-mouse secondary antibody (Cappel). Staining with an antibody against the cytoskeletal protein MAP2 (Amersham) was used as a control for the lack of permeabilization. Microscopical analysis was performed using an Axiophot microscope (Zeiss).

### 2.9. Immunoprecipitation

DRM raft fractions of stage 5 neurons were prepared as described before and solubilized by incubation at 37 °C for 30 min. Then samples were incubated in 1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 10 mM Tris pH 7.2, with a monoclonal antibody specific for γ enolase that does not recognize α enolase (Biogenesis) linked to protein G agarose beads (IP). An equivalent amount of DRM rafts was incubated with protein G agarose beads without the primary antibody (control).

### 2.10. Inhibition of plasminogen binding

GM1 in cultured hippocampal neurons was blocked by 5-min incubations with 12 μg/ml cholera toxin subunit B (chtx; Sigma) in Hanks' balanced salt solution (HBSS) containing 0.1% ovalbumin (Sigma) at 4 °C to avoid internalization, prior to the addition of human plasminogen (Sigma). In another set of experiments we added 100 U/ml carboxypeptidase B (cbB; Sigma) to the medium of hippocampal neurons for 30 min at 37 °C and 5% $CO_2$. After incubation with chtx or cbB, the cells were washed and 0.2 μM human plasminogen (Sigma) was added in HBSS containing 0.1% ovalbumin (Sigma). After 5 min at 37 °C samples were washed twice in HBSS for 5 min, extracted and analyzed by Western blot using a polyclonal antibody that recognizes human but not rat plasminogen (Biogenesis).

### 2.11. Inhibition of plasminogen binding after cholesterol depletion

Neurons were treated as indicated in Section 2.4 with 4 μM lovastatin and 0.25 mM mevalonate at day 5 in vitro. Neurons were grown for 4 more days and were then incubated with 5 mM methyl-β-cyclodextrin for 15 min at 37 °C. Then cells were washed and 0.2 μM human plasminogen was added in HBSS containing 0.1% ovalbumin. After 5 min at 37 °C, samples were washed twice in HBSS for 5 min, extracted and analyzed by Western blot using the polyclonal antibody against human plasminogen.

## 3. Results

### 3.1. The DRM raft fraction obtained from whole brain hippocampi contains proteins from different cell types

Previous work described the existence of neuronal rafts and their importance in axonal sorting [21,22] using primary cultures of rat hippocampal neurons. In order to characterize these membrane complexes in neurons, by defining their protein components, we conducted a first approach based on the use of adult rat hippocampal tissue, a source of similar cell types but in more abundant quantities. This would allow for the extraction of amounts of protein high enough to be identified by MALDI-TOF analysis of Coomasie Blue-stained bidimensional gels (2D). Triton X-100 detergent extraction and continuous sucrose gradient centrifugation [5] of hippocampal membranes led to the sequencing, in the raft fraction, of the Ras-GTPase-activating protein SH3-domain binding protein that participates in the ras signaling pathway consistently with the proposed role for rafts as signaling platforms [44]. However, glial myelin associated proteins such as the Myelin basic protein and the 1-2,3-cyclic nucleotide 3-phosphodiesterase and the specific mitochondrial molecules, NADH-ubiquinone dehydrogenase 24-kDa subunit and the F1-ATPase β subunit, were also detected. Thus, the use of whole hippocampal tissue as a source of membranes, together with DRM isolation based on continuous sucrose gradients, proved to be inadequate for precisely determining neuronal membrane raft proteins, due to the high levels of mitochondrial and glial contaminations.

### 3.2. Hippocampal neurons in primary culture are a good source for the identification of neuronal-specific raft proteins

To obtain a specific neuronal DRM fraction we used hippocampal neurons in primary culture under conditions that avoid glial proliferation [13] thus decreasing the possible glial contribution to negligible levels. In addition we used an Optiprep™-based step gradient (see Materials

and methods). This separation protocol reduced mitochondrial contamination to undetectable levels as determined using antibodies against mitochondrial markers (Fig. 1A). The specificity of the DRM raft interphase was further determined by Western blot analysis of the gradient fractions with antibodies against the raft and non-raft membrane markers flotillin 1 and transferrin receptor, respectively. Fig. 1A shows that while transferrin receptor is absent from the raft fraction flotillin 1 is enriched.

All optiprep fractions were also analyzed by 2D (Fig. 1B). For this purpose, primary cultures of mature hippocampal neurons were incubated with [$^{35}$S]methionine, detergent extracted and floated in Optiprep™ gradients to allow raft proteins move to the buoyant density of their associated lipids (30–5% Optiprep™ interphase). While most of the proteins remain in heavy fractions, a small percentage are present in the DRM raft density fraction. The specificity of this fraction was, as well, confirmed in 2D gels by pharmacological means reducing the levels of raft lipids in the cultured neurons before membrane purification (Fig. 2). An equivalent number of neurons was treated with Fumonisin B, which disrupts sphingolipid synthesis or with methyl-β-cyclodextrin, which depletes cholesterol from the cellular membrane (see Materials and methods). The efficiency of these treatments in cultured hippocampal neurons was previously demonstrated by

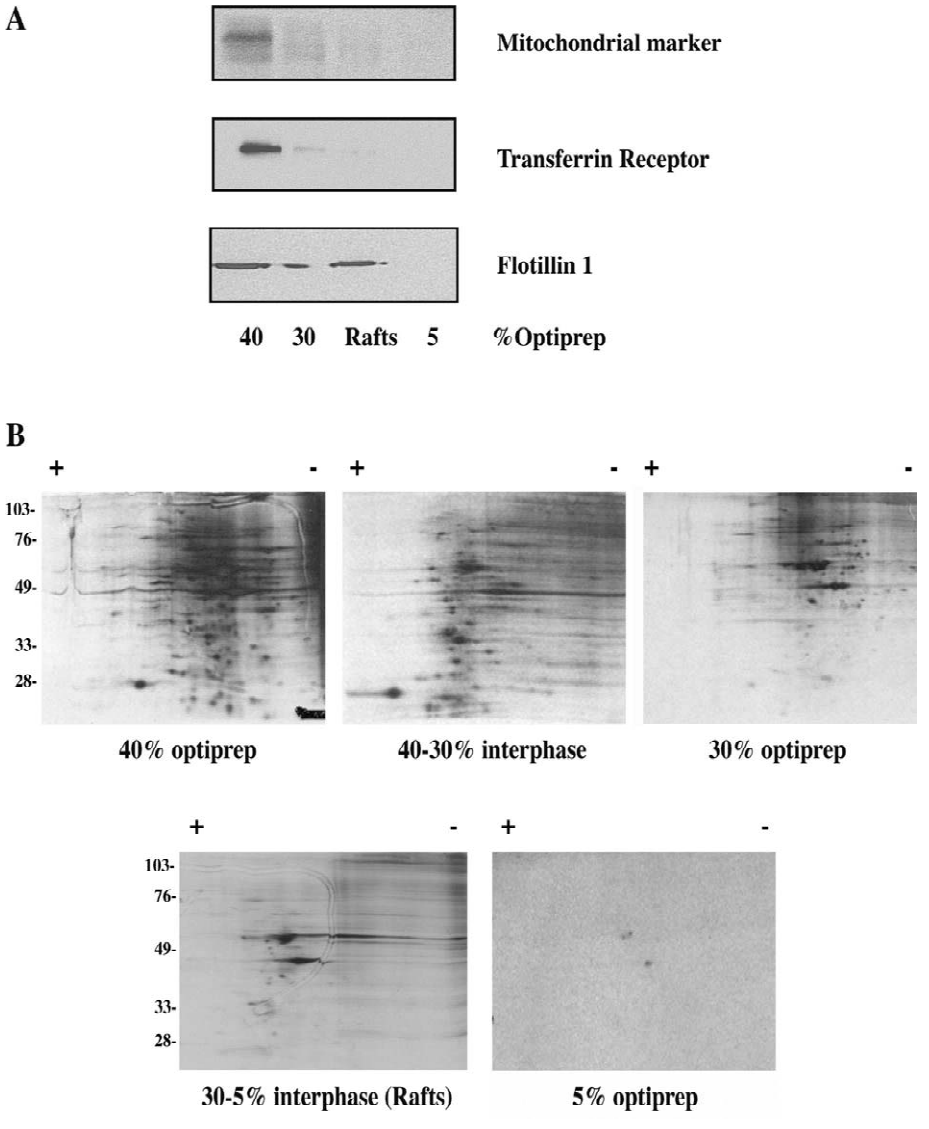


Fig. 1. Isolation of DRM raft fractions from primary hippocampal neurons using Triton X-100 extraction on ice and optiprep gradients. (A) Western blots of optiprep fractions (40%, 30%, interphase 30–5% (rafts) and 5%) obtained from hippocampal neurons after detergent extraction on ice, using antibodies against a mitochondrial marker, transferrin receptor and flotillin 1. While the mitochondrial marker and the non-raft marker transferrin receptor remain in heavy fractions, the known raft marker flotillin 1 is enriched in the DRM raft fraction. These results confirm the absence, in the DRM raft fraction, of mitochondrial contamination and indicate its specific raft nature. (B) Autoradiographs of the bidimensional gels corresponding to all optiprep fractions (40%, interphase 40–30%, 30%, rafts and 5%) from metabolically labeled mature hippocampal neurons. Molecular weights are shown on the left and the IEF direction is indicated on the top of each gel. While most of the proteins remain in heavy fractions only a minor pool appears in DRM rafts.

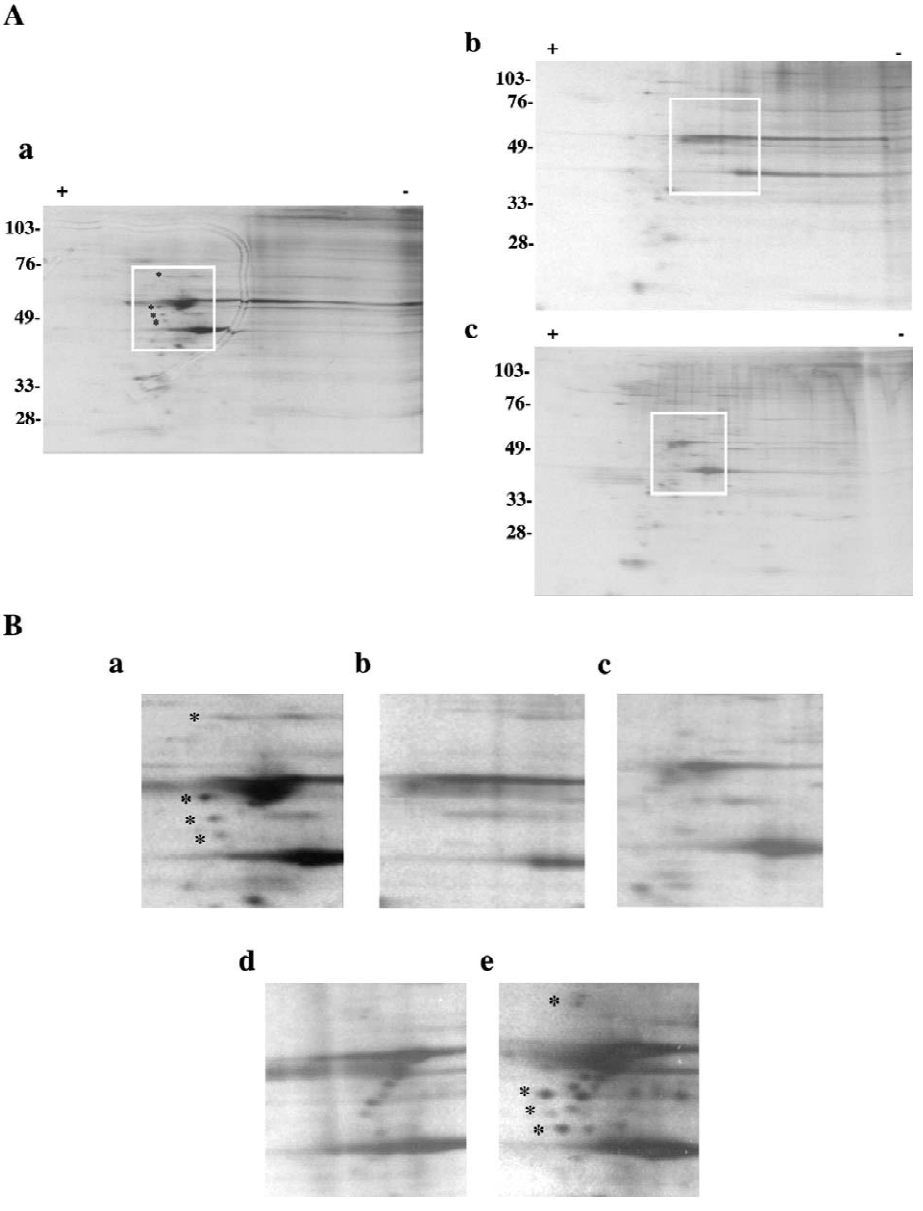


Fig. 2. 2D analysis of hippocampal neuronal DRM rafts: determination of raft specificity by lipid manipulation. (A) (a) Autoradiography of the bidimensional gel corresponding to DRM rafts from metabolically labeled mature hippocampal neurons. (b,c) Bidimensional gel pattern of the raft fraction from equivalent amount of metabolically labeled mature neurons treated with Fumonisin B or with methyl-β-cyclodextrin, respectively. The time of autoradiography exposure is the same for all samples. Notice the reduction of total protein levels after raft lipid inhibition. The spots indicated with an asterisk are those that presented a more drastic change, disappearing in the treated samples. White squares distinguish areas magnified in panel B. Molecular weights are shown on the left and the IEF direction is indicated on the top of each panel. (B) Insets from the areas defined in A are magnified to better illustrate the drastic decrease of marked spots (*) after inhibition of sphingolipids (using Fumonisin B) (b) or cholesterol (using methyl-β-cyclodextrin) (c) as compared to control samples (a). The lower panel shows the insets of equivalent areas from autoradiographies of 2D gels obtained with same number of immature neurons non treated (d) or treated (e) with NBD-C6-Ceramide, a sphingomyelin precursor. The marked (*) spots not present in the floated material from immature neurons that have a deficiency in rafts (a) appear in the ceramide treated neurons induced to form rafts (e). Notice that the marked spots correspond to those disappearing in raft depleted mature neurons (see a,b,c).

changes in detergent solubility of raft markers and alteration of raft-dependent polarized sorting [21]. Moreover, cholesterol removal was further monitored by fluorescence microscopy using filipin, a fluorescent polyene antibiotic that binds to cholesterol (not shown but see Ref. [45]). After the treatments neurons were radiolabeled and detergent extracted at 4 °C. DRM raft fractions were isolated in Optiprep™ step gradients and analyzed on 2D gels. The pattern obtained (Fig. 2Ab,c) was compared with that of same number of untreated neurons (Fig. 2Aa). Both sphingolipid and cholesterol reduction induced a similar decrease of raft protein content that for some peptides was drastic (marked with an asterisk in Fig. 2Aa). This is illustrated in the magnified examples in Fig. 2Ba–c. To

further confirm specificity an extra experiment was performed using immature hippocampal neurons, which have low levels of sphingomyelin and thus a low number of sphingolipid-cholesterol membrane microdomains [22]. Indeed, DRMs of immature neurons appear devoid of the same spots that disappear from the rafts of mature neurons in which sphingomyelin and cholesterol were reduced (Fig. 2Bd, compare with Fig. 2Ba). Since rafts can be induced in immature neurons by adding a precursor of sphingomyelin, NBD-Ceramide [22], we analyzed DRMs of ceramide-treated immature neurons. The result of this rescue experiment is shown in Fig. 2Be. Proteins normally not present in the DRM raft fraction of immature neurons (Fig. 2Bd) appeared after the addition of NBD-Ceramide (Fig. 2Be). These are the same as those previously shown to be drastically affected by both Fumonisin B and methyl-β-cyclodextrin treatments of the fully mature neurons (Fig. 2Bb,c) confirming their strict raft association.

### *3.3. Raft protein sequencing analysis*

Having determined the specificity of the proteins in our DRM raft fraction in a bidimensional gel pattern of metabolically labeled neurons, we moved on to scale up our experimental system so as to obtain a Coomasie Blue-stained replica of the previously shown gel (Fig. 2Aa). Such gel would provide sufficient amounts of protein to perform MALDI-TOF protein mapping. DRM raft material from 100 dishes with hippocampal neurons (150 000 cells/dish) was prepared. The pattern obtained was very similar when compared to the gel with radiolabeled material (Fig. 3A,B). Three out of the four spots, previously identified as drastically affected by the lipid manipulation, were visible. Sequencing of these spots, numbers 1, 5 and 6, identified them as the heat shock protein 70, ATP synthase β chain and the neuronal specific enolase (γ enolase), respectively. Spots numbers 2, 3, 4 (which correspond to a doublet) and 7, also enriched in the neuronal raft fraction, were identified as Tubulin α1, α6, α2 and β5 chains and β actin, respectively.

Proteins associated with sphingolipid-cholesterol complexes are membrane bound making them difficult to handle. This causes the lipid content of the loading samples to be extremely high in comparison with the amount of protein, causing technical problems particularly in bidimensional gel protocols. The use of a methanol/chloroform delipidation protocol for sample concentration [49] together with unidimensional SDS–PAGE analysis, that allows higher temperatures and higher levels of detergent, helped us to isolate other proteins while reducing the lipid content versus protein amount ratio, as well as the levels of protein aggregation. Reliability was assured, as the molecular weights of interesting spots were known. Moreover, the very abundant, recurring spots visible on the 2D gels (identified as 3, 4 and 7 in Fig. 3A,B) were likely to be represented by thick bands in a 1D gel. These thick bands were indeed present (Fig. 3C) and their sequence was confirmed by MALDI-TOF serving as a reference point for the identification of bands corresponding to the spots of interest. This analysis allowed for the partial

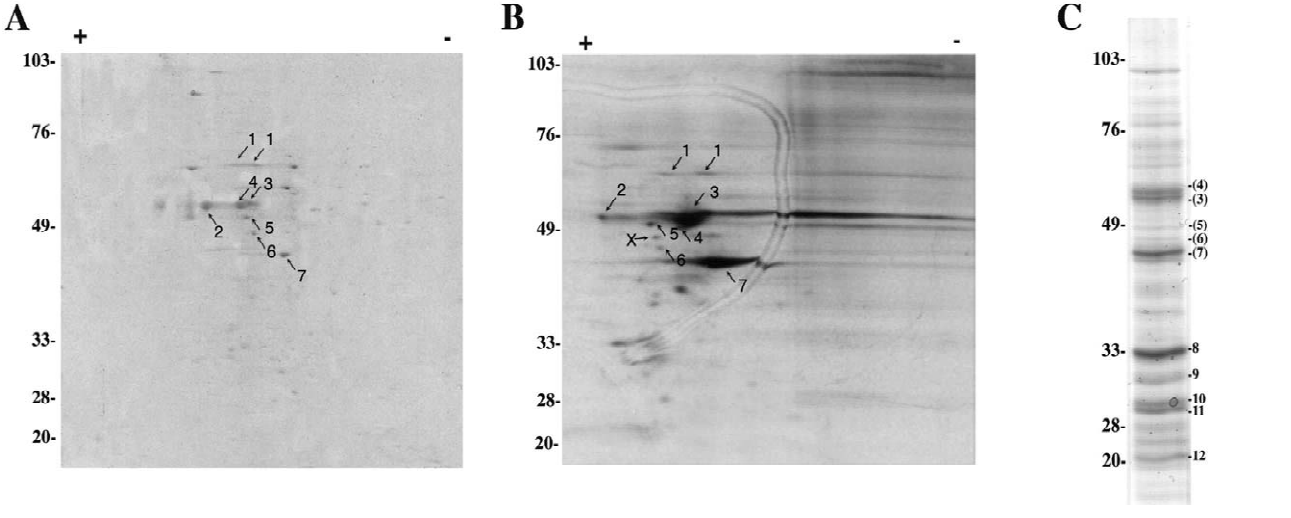


Fig. 3. Raft protein sequences. (A) Coomasie Blue stained replica of the 2D gel autoradiography shown in Fig. 1A and depicted in panel B. Spots marked with numbers (1–7) in panel A are those selected for sequencing. The sequenced spots have their correspondence indicated in panel B. Spots 1, 5 and 6 are drastically affected by the manipulation of raft lipids together with spot X detected in the autoradiographic analysis but not obtained in the Coomasie gel. IEF direction is indicated on top of the gels. Molecular weights are shown on the left. The sequences obtained corresponded to: 1, Hsp70; 2, Tubulin α1 chain; 3, Tubulin α6 chain; 4, (corresponding to a doublet) Tubulin α2 and β5 chains; 5, ATP synthase β chain; 6, γ enolase; and 7, β actin. (C) 1D gel stained with Coomasie blue from the DRM raft fraction of mature hippocampal neurons. Bands indicated with numbers were those selected for sequencing. Bands numbered between brackets are equivalent to the sequenced spots from the 2D gel analysis (see panel A). Molecular weights are shown on the left. The new sequences obtained corresponded to: 8, α enolase; 9, ATP synthase α chain; 10, ADP/ATP carrier protein; 11, ADP/ATP carrier protein; and 12, Thy-1 membrane glycoprotein.

determination of more raft-associated proteins like α enolase, ATP synthase α chain, ADP/ATP carrier proteins and the known raft marker [21] Thy-1 membrane glycoprotein (numbers 8, 9, 10, 11 and 12, respectively).

### *3.4. The neuronal specific enolase (γ enolase) interacts with the α enolase in rafts of hippocampal neurons*

From the proteins identified as raft components, γ enolase (spot number 6; Fig. 3) is neuronal specific opening new perspectives for raft function in neurons. This is a cytosolic enzyme that participates in the glycolytic chain [29]. Moreover, it has also been described to be present in the plasma membrane [29]. This was confirmed by surface staining using a specific antibody against γ enolase that revealed clustering of the protein, in discrete spots, at the surface of mature hippocampal neurons (Fig. 4A). Enolases are active as dimers and are present in three forms (γ-γ, α-γ and α-α) in adult rat brain extracts [18]. Indeed, one of the partial sequences obtained from the 1D gel analysis of DRM raft fractions corresponded to α enolase. To confirm the presence of this isoform in rafts and to establish whether it interacts with γ enolase in hippocampal neurons, we performed immunoprecipitations of DRM fractions using a specific antibody against γ enolase as shown in Fig. 4Ba. The immunoprecipitated material was then probed with a specific antibody against α enolase. The α enolase was brought down by the anti γ enolase antibody indicating its presence in rafts together with the γ isoform forming heterodimers or even oligomers as suggested by the detection of high molecular weight bands in Western blots of DRM raft total fractions using both anti α and anti γ enolase antibodies (Fig. 4Bb).

### *3.5. Enrichment of plasminogen binding molecules and specific plasmin activation in rafts of hippocampal neurons*

The C-terminal lysines of α enolase are a prominent binding site for the kringle domains of plasminogen [28,29]. Not only α-enolase but also other C-terminal containing lysine proteins such as annexin II and amphoterin, as well as the ganglioside GM1, have also been related to plasminogen binding in different cell types [15,27,34]. Thus, we tested whether these plasminogen binding molecules are also enriched in rafts of hippocampal neurons. Mature neurons in culture were extracted in Triton X-100 on ice and floated in an Optiprep™ gradient. We analyzed each of the fractions from the gradients by slot-blot using cholera toxin peroxidase linked to detect GM1 or by Western blot using specific antibodies against α enolase, annexin II and amphoterin. All four molecules appeared enriched in the membrane fraction corresponding to DRM rafts (Fig. 4C).

To test the functional significance of the enrichment of these molecules in the DRM raft fraction in plasminogen binding, we performed the following experiment. Hippocampal neurons were incubated with cholera toxin subunit B (chtx) and/or carboxypeptidase B (cbB) that specifically block GM1 and remove exposed C-terminal lysines, respectively [19,28]. The treatments were followed by incubation with human plasminogen and then by analysis of plasminogen binding. Chtx and cbB decreased binding by up to 38 and 40%, respectively. The simultaneous addition of chtx and cbB led to the almost complete elimination of plasminogen binding (Fig. 4D). Binding to the membrane is an obligatory step in the conversion of plasminogen into plasmin [37]. The above reported enrichment of plasminogen binding molecules in the raft fraction suggested that plasmin conversion must occur preferentially in rafts of these cells. Indeed, in Fig. 4E we show that plasmin conversion is restricted to the raft fraction of the neuronal membrane.

### *3.6. Raft disruption inhibits plasminogen binding and conversion into plasmin*

To further analyze the requirement of rafts for plasminogen binding and activation, we disrupt these microdomains by extracting membrane cholesterol from hippocampal neurons. Cells were treated or not with methyl-β-cyclodextrin (see Materials and methods). Under the conditions used up to 60% of membrane cholesterol is extracted [45] and both detergent insolubility and sorting of raft proteins are altered [21]. Human plasminogen was then added to the neuronal cultures and the amount of plasminogen bound and plasmin produced detected by Western blot (Fig. 4Eb). Both events were significantly reduced after raft disruption confirming the requisite of intact rafts.

## 4. Discussion

In this work we have set up a protocol to purify neuronal DRM raft fractions from primary hippocampal neurons. The specificity of our approach is demonstrated by the presence of specific raft markers (flotillin 1, Thy-1, GM1), the absence of non-raft markers (transferrin receptor) and by pharmacological means. We were able to scale up the protocol in order to obtain sufficient amounts of raft proteins for their MALDI-TOF characterisation.

The sequencing of Thy-1, a known raft protein that requires raft association to be sorted to the axonal membrane of hippocampal neurons [21], not only indicates that the floated material used indeed corresponds to a raft-enriched fraction but also underscores the first physiological role assigned to these complexes as part of the membrane protein sorting machinery. Our results also revealed an association of rafts with cytoskeletal components such as tubulin and actin. Although a proportion of them could be due to contamination given the high

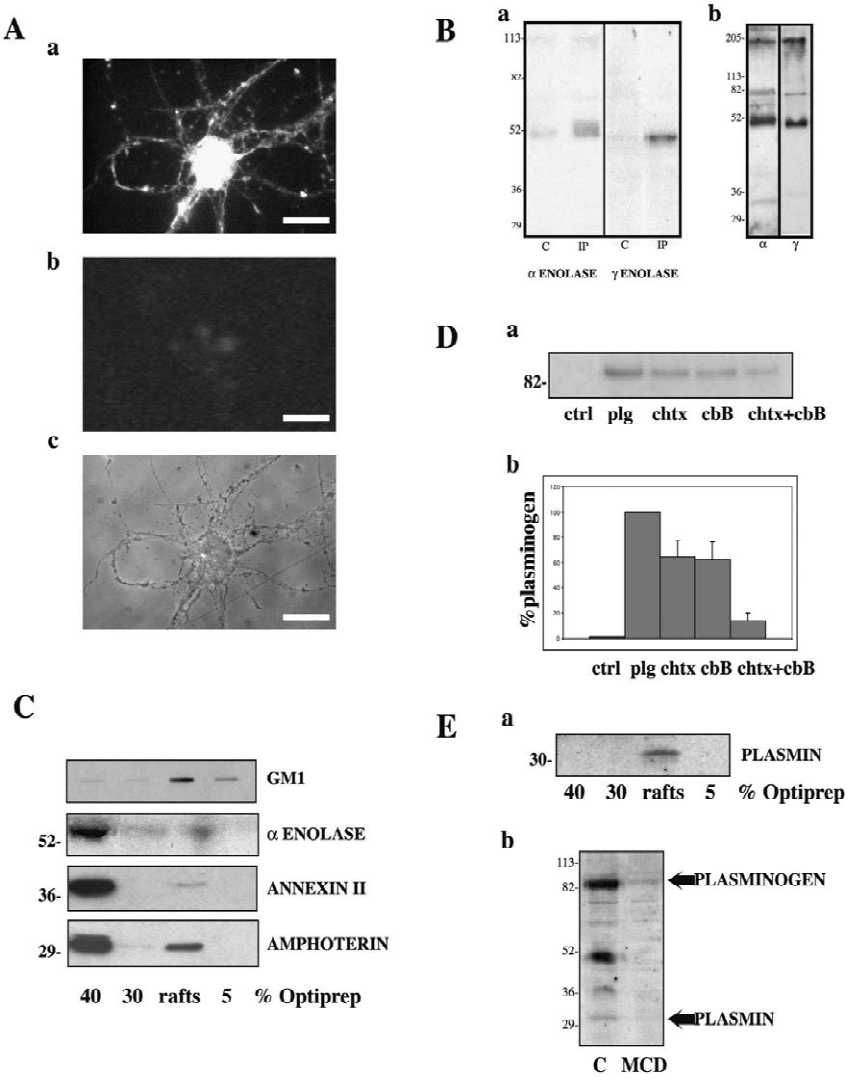


Fig. 4. Enrichment of plasminogen binding molecules and exclusive plasmin activation in rafts. (A) γ Enolase is at the surface of mature hippocampal neurons. (a) Surface staining using a specific antibody against γ enolase of a mature hippocampal neuron. (b) Absence of staining with an antibody against the cytoskeletal protein MAP2 used as a control to rule out the possibility of cell permeabilization during the fixation protocol. (c) Phase contrast. Scale bar represents 10 μm. (B) γ Enolase interacts with α enolase in rafts of hippocampal neurons. (a) The γ enolase antibody linked to protein agarose beads was used to immunoprecipitate DRM raft fractions from mature hippocampal neurons (IP). Equivalent amounts of DRM raft fractions were incubated with the protein G agarose beads alone (C). Western blot of these samples with the anti γ enolase antibody used to immunoprecipitate shows the antibody specificity (right panel, γ enolase). Western blot of these samples using the α enolase antibody confirmed the interaction of α and γ enolases in rafts (left panel, α enolase). (b) Western blots of total DRM raft fractions from hippocampal neurons using specific antibodies against α enolase or γ enolase. High molecular weight bands are detected by both antibodies most probably corresponding to heterodimers (82 kDa) and heterotetramers (205 kDa). (C) C-terminal lysine containing proteins and GM1 are enriched in the raft membrane fraction of hippocampal neurons. Mature rat hippocampal neurons in culture were extracted in Triton X-100 on ice and floated in an Optiprep™ gradient. We analyzed equal volumes of each of the gradient fractions (40%, 30%, interphase 30–5% and 5%) by slot-blot using cholera toxin peroxidase linked to detect GM1 or by Western blot using specific antibodies against α enolase, annexin II and amphoterin. All three proteins appeared in the non-floated 40% fraction containing cytosolic proteins but were also enriched in the membrane fraction corresponding to rafts (interphase 30–5% Optiprep™) as well as the ganglioside GM1. Molecular weights in kDa are indicated on the left. (D) C-terminal lysine containing proteins and GM1 mediate the binding of plasminogen to hippocampal neurons. Human plasminogen was added to primary cultures of rat hippocampal neurons treated or not with cholera toxin subunit B (chtx) to specifically block GM1 and/or with carboxypeptidase B (cbB) to remove exposed C-terminal lysines. (a) Using a specific antibody against human plasminogen the following samples were analyzed by Western blot: rat hippocampal neurons (ctrl); rat neurons incubated with human plasminogen (plg); rat neurons treated with 12 μg/ml chtx B and human plasminogen (chtx); rat neurons treated with 100 U/ml of cbx B and human plasminogen (cbB) and rat neurons treated with both chtxB and cbx B and human plasminogen (chtx+cbB). Plasminogen binding was reduced up to 38% after Chtx treatment and 40% upon cbx incubation. The simultaneous addition of chtx and cbB led to the almost complete elimination of plasminogen binding. Molecular weight in kDa is indicated on the left. (b) Graph represents mean values with standard deviations of the quantification of the Western blots, as in Da, corresponding to three different experiments. Values are indicated as percentage of plasminogen binding in comparison to the plg sample (100%). (E) Plasminogen binding and activation requires intact rafts. (a) Plasmin is exclusively present in the raft fraction of hippocampal neurons. Mature rat hippocampal neurons were detergent extracted on ice and floated in an optiprep gradient. The figure shows the Western blot of all gradient fractions using an antibody against rat plasminogen that also recognizes the 30-kDa plasmin fragment. Molecular weight is indicated on the left. (b) Raft disruption inhibits plasminogen binding and activation into plasmin. Mature rat hippocampal neurons were treated (MCD) or not (C) with methyl-β-cyclodextrin that extracts membrane cholesterol. Human plasminogen was then added to the cells and the amount of plasminogen bound and plasmin produced (indicated by arrows) analyzed by Western blot. Molecular weight markers are indicated on the left.

amount of these proteins in the cells, the fact that their levels in rafts are significantly reduced after raft lipid inhibition made us believe they are truly associated to these microdomains at least in part. Moreover, the presence of lipid-anchored tubulin within detergent resistant domains has already been described [33]. This localization could drive structural remodeling of the plasma membrane and/or mediate signal transduction involving mechanisms relying on the GTP-binding protein properties of tubulin [14,38]. This, together with the fact that in previous attempts to identify neuronal raft associated proteins other signaling molecules such as Src family protein tyrosine kinases (c-Src, Lyn and Fyn) were identified [39], further supports the role of rafts in signal transduction. On the other hand, the presence of actin binding proteins has been reported in rafts and the association with actin has been hypothesized as a way to control raft dynamics at the surface of different cell types [1,20,30,32,48]. The rafts themselves would generate the energy required for such events. Indeed, we find a number of energy related molecules in our DRM complexes. We identified not only molecules capable of synthesizing ATP (ATP synthase) but also of transporting and exchanging it (ADP/ATP carriers). This may indicate that rafts need to have readily available ATP molecules to be used by raft-associated proteins. One such protein could be hsp70. This is an enzyme that solubilizes and refolds denatured proteins via cycles of ATP binding and hydrolysis [11]. The fact that this is one of the spots that showed strict DRM raft specificity, totally disappearing when the raft lipids were decreased, indicates that it is a true raft protein and not a contamination due to cellular stress-induced overexpression. Hsp70 has been shown to be neuroprotective in the nervous system [16,42]. Imbalances in the amounts of certain proteins, or their incorrect processing, potentially endanger the nervous system developmental process and are the cause of many neurological conditions. It would thus be reasonable to consider that rafts may play a role in neuroprotection.

We have detected enolases in raft membrane domains and at the neuronal cell surface. How these proteins, first identified in the cytosol and that do not contain a signal peptide for classical secretion, reach the extracellular space is yet to be determined. In this regard, alternative mechanisms have been proposed for the unconventional protein secretion of proteins such as interleukin-1β, growth factors FGF-1 and FGF-2 or galectins [31]. In the work presented here, not only α enolase was identified in rafts, but also molecules such as annexin II, amphoterin and GM1 that have been described to bind plasminogen in different cell types, indicating a crucial role for these microdomains in the plasminogen activation system. Our biochemical work with specific inhibitors confirmed that these molecules are mediating the binding of plasminogen in hippocampal neurons, therefore explaining the raft-specific activation to the proteolytically active fragment plasmin in these microdomains (Ref. [23] and this work). We also show that raft integrity is required for these events. There is accumulating evidence that plasminogen activated to plasmin regulates local degradation/stabilisation of the extracellular matrix thus influencing neuronal plasticity [6]. By virtue of its role, this protease has been directly involved with essential neuronal events such as neuronal migration and pathfinding [41], neurite outgrowth [36], and synaptic growth [3], as well as in plasticity changes necessary for LTP (long-term potentiation) generation and therefore in memory acquisition [3,24].

Altogether, we believe the data described here open new perspectives for the role of sphingolipid-cholesterol microdomains in the biology of neurons.

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